

## Rapid Identification of Bacteria on the Basis of Polymerase Chain Reaction-Amplified Ribosomal DNA Spacer Polymorphisms

MARK A. JENSEN,<sup>1\*</sup> JOHN A. WEBSTER,<sup>1</sup> AND NEIL STRAUS<sup>2</sup>

Central Research and Development Department, E. I. du Pont de Nemours & Company (Inc.), Wilmington, Delaware 19880,<sup>1</sup> and Department of Botany, University of Toronto, Toronto, Ontario, Canada M5S 3B2<sup>2</sup>

Received 23 July 1992/Accepted 18 January 1993

To facilitate genus and species level identification of a broad range of bacteria without the requirement of presumptive identification, we have developed a unified set of primers and polymerase chain reaction conditions to amplify spacer regions between the 16S and 23S genes in the prokaryotic rRNA genetic loci. Spacer regions within these loci show a significant level of length and sequence polymorphism across both genus and species lines. A generic pair of priming sequences was selected for the amplification of these polymorphisms from highly conserved sequences in the 16S and 23S genes occurring adjacent to these polymorphic regions. This single set of primers and reaction conditions was used for the amplification of 16S-23S spacer regions for over 300 strains of bacteria belonging to eight genera and 28 species or serotypes, including *Listeria*, *Staphylococcus*, and *Salmonella* species and additional species related to these pathogenic organisms. When the spacer amplification products were resolved by electrophoresis, the resulting patterns could be used to distinguish all of the species of bacteria within the test group. Unique elements in the amplification product patterns generally clustered at the species level, although some genus-specific characteristics were also observed. On the basis of the results obtained with our test group of 300 bacterial strains, amplification of the 16S-23S ribosomal spacer region is a suitable process for generating a data base for use in a polymerase chain reaction-based identification method, which can be comprehensively applied to the bacterial kingdom.

The rRNA genetic locus, *rnm*, is a genetic unit of broad evolutionary interest since it is found in both prokaryotic and eukaryotic organisms. In fact, there is sufficient conservation within this locus for it to be used in a universal organization of evolutionary relationships (5). The utility of the rRNA sequence as a taxonomic tool has been amply demonstrated in bacteria, where 16S rRNA sequence analyses have completely redefined phylogenetic relationships previously too dependent on cellular metabolism (7, 17, 26, 27). In addition to highly conserved areas that have been used to study the relationships among distant taxa, the 16S sequence contains more variable regions that have been useful in the differentiation of genera and species (10). This differentiation has been accomplished through the use of probes which have been generated by using conserved 16S sequences as universal primers for polymerase chain reaction (PCR) amplification (22) of certain variable 16S regions (2). The conserved nature of the rRNA sequences has also led to the development of generic DNA probes for bacteria. These probes have been used to visualize specific components of restriction-digested bacterial genomic DNA, which have been separated by electrophoresis. The resulting rRNA gene restriction patterns have been used for the differentiation of species and strains within a given genus and as a generic tool for the identification of a broad range of bacteria (11, 21, 23, 25).

In prokaryotes, the rRNA genetic loci contain the genes for all three rRNA species, 16S, 23S, and 5S genes. These genes are separated by spacer regions which exhibit a large degree of sequence and length variation at the levels of genus and species. Within a single genome there are frequently

multiple rRNA genetic loci; spacer regions found within these loci also show a significant degree of variation in length and sequence. This diversity is due in part to variations in the number and type of tRNA sequences found within the spacers (4, 19). Recently, it has been shown that length and sequence polymorphisms in the spacers within the *rnm* loci can be used to discriminate between different species of prokaryotes (1). Spacer region polymorphisms have also been useful in the species identification of several fungi (8, 24).

The most direct and certainly the most rapid method to visualize the polymorphic character of internal *rnm* spacers is to carry out PCR amplification of the spacer regions by using primers from highly conserved flanking sequences. The length and sequence polymorphisms present in the PCR product can then be used in the recognition of genera and species. Additional information inherent in the polymorphic character of the amplified product is accessible by several means. The PCR product can be digested with a restriction enzyme, and the resulting fragments can be resolved electrophoretically. If the PCR product contains the restriction endonuclease recognition sequence at unique locations, then the resultant fragment size pattern can be indicative of a particular species (8, 24). A second approach is to use DNA probes which contain a sequence which is unique to the target of interest. The hybridization of the PCR product with such a probe can be used to identify the target organism (1, 2). A third approach is to carry out the DNA amplification by using primer sequences and amplification conditions which discriminate for the organism of interest. If the size of the spacer region(s) is known to be unique, then the presence of such amplification products is diagnostic for the presence of the target organism.

In this report we describe a unified set of primers and PCR

\* Corresponding author.

conditions which can be used to amplify spacer regions between the 16S and 23S genes of the rRNA genetic loci. The primers and amplification conditions can be applied to DNAs isolated from a wide variety of bacterial samples without the necessity of designing special assay conditions for particular groups of bacteria. The amplification primer sequences were selected from regions of highly conserved sequences found in the 16S and 23S regions within 40 bases of the intervening spacer region (see Fig. 1). PCR amplification of the *rrn* spacer regions from these conserved sites produced DNA fragments whose sizes and numbers could be used to rapidly identify a broad range of bacteria.

## MATERIALS AND METHODS

**DNA amplification.** High-molecular-weight, bacterial genomic DNA was purified from cell lysates by two sequential chloroform-phenol extraction and ethanol precipitation steps. The DNA was redissolved in a buffer containing 10 mM Tris and 1 mM EDTA, and its concentration was determined spectrophotometrically. DNA samples were diluted to a concentration of 20 ng/ $\mu$ l prior to amplification. A 1.25- $\mu$ l aliquot of bacterial genomic DNA was combined with 2.5  $\mu$ l of reaction buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.8 at 25°C], 15 mM MgCl<sub>2</sub>, 1% Triton X-100), 1  $\mu$ l of a deoxynucleoside triphosphate (dNTP) mixture (concentration of each dNTP, 5 mM), 1.25  $\mu$ l each of two 15-base oligonucleotide primers (primers G1 and L1 [concentration, 50 ng/ $\mu$ l]), and 42  $\mu$ l of deionized water. This mixture was heated to 94°C for 5 min, and 1.3 U of a thermostable DNA polymerase was added. Twenty-five amplification cycles were performed with an automated thermocycler according to the following format: 1 min at 94°C; 2-min ramp to 55°C; 7 min at 55°C; 2-min ramp to 72°C; and 2 min at 72°C. The final cycle was followed by an additional 7 min at 72°C to complete partial polymerizations. At the end of the cycling program 1  $\mu$ l of 0.5 M EDTA was added as a stop solution, and the products were stored at 4°C.

**Electrophoresis and imaging.** A 5- $\mu$ l aliquot of the reaction mixture was combined with 2  $\mu$ l of loading buffer (15% Ficoll, 0.25% xylene cyanol), and the preparation was electrophoresed on a 4% acrylamide-bis (29:1) gel. The gels were stained with ethidium bromide and photographed on a UV transilluminator.

**Determination of PCR product sizes.** Molecular weight markers were constructed by amplifying regions having known lengths and sequences from the *rrnB* rRNA operon of *Escherichia coli* (4). Molecular weight markers were run in the edge lanes and every third lane in the internal portion of the gel. Every lane containing amplification products from genomic bacterial DNA was run adjacent to a lane containing molecular weight markers. The sizes of the fragments produced in the amplifications were calculated from the positions of these fragments relative to the positions of the molecular weight markers in the adjacent lanes. The level of uncertainty in the calculated sizes of the amplification products was approximately 2%.

**Identification of bacteria strains.** Bacterial strains were identified by observations of colonial morphology and Gram staining and by well-established biochemical tests. In addition, genomic DNA was used to generate a ribotyping data base which was also used to confirm the species identification of all bacteria in the test panel (11, 25).

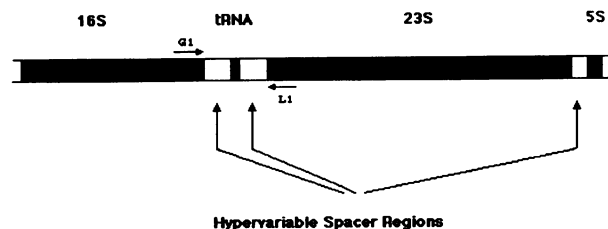


FIG. 1. Schematic representation of an rRNA operon showing the approximate priming sites for the PCR amplification of the 16S-23S spacer region. The number of tRNA genes in this spacer region is subject to some variation.

## RESULTS

**Amplification primers and reaction conditions.** The 16S regions of the *rrn* locus have been sequenced for a broad range of bacteria (6). The first primer, G1, was selected from a highly conserved region immediately adjacent to the 16S-23S spacer. This oligonucleotide contains the sequence GAAGTCGTAACAAGG and is located about 30 to 40 nucleotides upstream from the spacer boundary (Fig. 1). The second primer, L1, was selected from the five bacterial and four plant chloroplast 23S sequences compiled by Gutell and Fox (12). This sequence, CAAGGCATCCACCGT, is the most conserved 23S sequence immediately following the spacer and is located about 20 bases downstream from the spacer boundary (Fig. 1). Primers for both the 16S and 23S regions were limited to a length of 15 bases because of variations in sequence beyond these highly conserved regions.

Since the area targeted for the amplification reaction is known to exhibit a high degree of single-stranded secondary structure which can compete with primer hybridization, we modified the PCR reaction conditions from those typically used (13). The principal modification consisted of the use of a substantially longer primer annealing time, 7 min, along with very high stringency annealing conditions, 55°C. The 55°C annealing temperature was the highest temperature at which amplification products were reproducibly formed with 15-base primers. In the amplification reaction solution the dNTP, Mg, KCl, primer, and polymerase levels selected fell at the low end of the functional amplification range: [dNTP], 0.1 mM; [Mg<sup>2+</sup>], 0.75 mM; [KCl], 25 mM; [primer], 0.25  $\mu$ M; and polymerase, 1.3 units. Under these amplification conditions 25 cycles were required to generate reproducible and easily detectable product profiles. Similar profiles were also produced by using the same reaction conditions for 20 cycles; however, for comparable results the annealing time had to be increased to 10 min. The patterns resulting from these reaction conditions could be easily analyzed and processed by the types of pattern recognition software which are used for comparisons with a reference pattern data base. Use of higher numbers of cycles and shorter annealing times resulted in significantly more complex patterns in which the putative *rrn* spacer fragments were present only as weak secondary bands. Such reactions also contained significantly higher levels of nonspecific amplified DNA product, which substantially increased the background.

**Amplification product profiles.** Amplifications of the spacer regions between the 16S and 23S rRNA genetic loci were run for over 300 strains of bacteria representing eight different genera. Multiple species belonging to the genera *Listeria* and *Staphylococcus* and multiple serotypes belonging to the genus *Salmonella* were chosen since these taxa represent a

TABLE 1. Number of strains studied for each species

Species	No. of strains	Species	No. of strains
<i>L. monocytogenes</i>	68	<i>Enterobacter agglomerans</i>	2
<i>L. welshimeri</i>	9	<i>Enterobacter cloacae</i>	16
<i>L. innocua</i>	15	<i>Enterobacter aerogenes</i>	4
<i>L. ivanovii</i>	10	<i>Staphylococcus aureus</i>	29
<i>L. murrayi</i>	2	<i>Staphylococcus saprophyticus</i>	8
<i>Salmonella typhimurium</i>	10	<i>Staphylococcus warneri</i>	5
<i>Salmonella enteritidis</i>	4	<i>Staphylococcus epidermidis</i>	5
<i>Salmonella newport</i>	2	<i>Escherichia coli</i>	95
<i>Salmonella infantis</i>	3	<i>Escherichia fergusonii</i>	1
<i>Salmonella typhi</i>	2	<i>Escherichia vulneris</i>	1
<i>Salmonella choleraesuis</i>	1	<i>Escherichia hermani</i>	1
<i>C. freundii</i>	14	<i>Escherichia blattae</i>	1
<i>C. diversus</i>	4	<i>P. mirabilis</i>	4
<i>Y. enterocolitica</i>	2	<i>P. vulgaris</i>	6

significant number of pathogenic microorganisms whose identification and characterization is particularly important. Five species were examined from the genus *Escherichia*, and eight additional species were examined from four genera which are related to the pathogenic species of interest. Table 1 summarizes the composition of the data base from which examples of amplification product profiles are shown.

The patterns shown in Fig. 2 through 6 are representative samples of the product profiles generated by amplification of the 16S-23S spacer region for the bacterial species shown in Table 1. To facilitate comparisons of the PCR product profiles, the amplification products are classified in two groups. The intense and highly reproducible fragments are referred to as primary products. The weaker or more variable fragments are referred to as secondary products. A summary of primary and secondary PCR products is provided in Table 2.

**Listeria species.** Figure 2 shows the patterns of products from the amplification of the 16S-23S spacer region for five species belonging to the genus *Listeria*. The four strains of

*Listeria monocytogenes* in Fig. 2 each contained the same pair of primary fragments at 355 and 620 bp. These two fragments were observed in each of the 68 strains of *L. monocytogenes* in the data base (Table 1). Two additional weak bands were also occasionally observed for *L. monocytogenes*. Eleven strains showed a faint 560-bp fragment, and five strains showed a very faint fragment at 770 bp. The two strains of *Listeria welshimeri* in Fig. 2 showed primary fragments at 355 and 635 bp. These two fragments were common to each of the nine strains of *L. welshimeri* in the data base. An additional very weak band at 825 bp was also observed for two strains of *L. welshimeri*. Both strains of *Listeria innocua* showed fragments at 355 and 655 bp. These fragments were common to each of the 15 strains of *L. innocua* in the data base. The two strains of *Listeria ivanovii* in Fig. 2 showed fragments at 380 and 605 bp. These two fragments were observed for each of the 10 strains of *L. ivanovii* in the data base. Both strains of *Listeria murrayi* showed fragments at 335 and 570 bp.

Although each *Listeria* species possesses a unique amplification product profile, the difference between the primary fragments sizes of *L. monocytogenes* and *L. welshimeri* is at

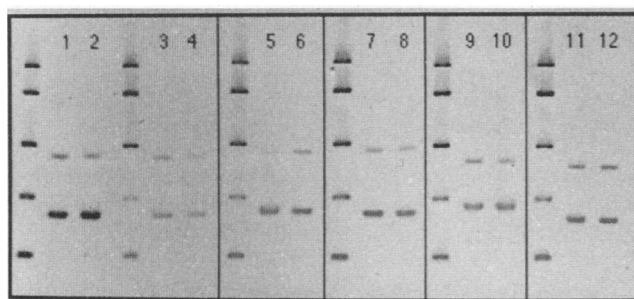


FIG. 2. Composite photograph showing PCR amplification products for the following bacterial genomic DNA samples: lane 1, *L. monocytogenes* 682; lane 2, *L. monocytogenes* 891; lane 3, *L. monocytogenes* 1149; lane 4, *L. monocytogenes* 1148; lane 5, *L. welshimeri* 1175; lane 6, *L. welshimeri* 1176; lane 7, *L. innocua* 644; lane 8, *L. innocua* 921; lane 9, *L. ivanovii* 1165; lane 10, *L. ivanovii* 1166; lane 11, *L. murrayi* 643; lane 12, *L. murrayi* 944. The unnumbered lanes contained molecular weight markers having the following sizes: 228, 412, 693, 1,331, and 2,306 bp. The PCR products were electrophoresed in 4% acrylamide-bis (29:1) by using a 0.5× Tris-borate-EDTA running buffer for 45 min at a field strength of 14 V/cm. Following electrophoresis the gels were stained for 15 min in a solution containing 0.25 µg of ethidium bromide per ml.

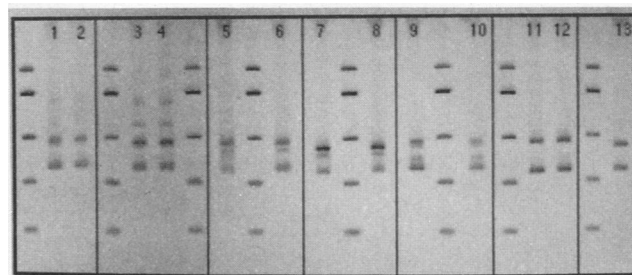


FIG. 3. Composite photograph showing PCR amplification products for the following bacterial genomic DNA samples: lane 1, *Salmonella typhimurium* 731; lane 2, *Salmonella typhimurium* 590; lane 3, *Salmonella typhimurium* 588; lane 4, *Salmonella typhimurium* 587; lane 5, *Salmonella enteritidis* 737; lane 6, *Salmonella enteritidis* 706; lane 7, *Salmonella newport* 735; lane 8, *Salmonella newport* 707; lane 9, *Salmonella infantis* 908; lane 10, *Salmonella infantis* 900; lane 11, *Salmonella typhi* 585; lane 12, *Salmonella typhi* 584; lane 13, *Salmonella choleraesuis* 917. The unnumbered lanes contained molecular weight markers having the following sizes: 228, 412, 693, 1,331, and 2,306 bp. The electrophoresis and staining conditions used are described in the legend to Fig. 2.

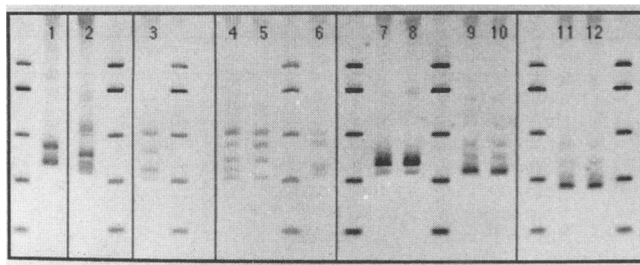


FIG. 4. Composite photograph showing PCR amplification products for the following bacterial genomic DNA samples: lane 1, *Staphylococcus aureus* 59; lane 2, *Staphylococcus aureus* 207; lane 3, *Staphylococcus aureus* 798; lane 4, *Staphylococcus aureus* 822; lane 5, *Staphylococcus aureus* 610; lane 6, *Staphylococcus aureus* 753; lane 7, *Staphylococcus warneri* 793; lane 8, *Staphylococcus warneri* 797; lane 9, *Staphylococcus saprophyticus* 866; lane 10, *Staphylococcus saprophyticus* 804; lane 11, *Staphylococcus epidermidis* 796; lane 12, *Staphylococcus epidermidis* 788. The unnumbered lanes contained molecular weight markers having the following sizes: 228, 412, 693, 1,331, and 2,306 bp. The electrophoresis and staining conditions used are described in the legend to Fig. 2.

the limit of the gel resolution. Other *Listeria* species can be readily distinguished from *L. monocytogenes* on the basis of the products generated from amplification of the 16S-23S spacer region.

**Salmonella serotypes.** Figure 3 shows the patterns of 16S-23S spacer amplification products for six serotypes belonging to the genus *Salmonella* (*Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella newport*, *Salmonella infantis*, *Salmonella typhi*, and *Salmonella choleraesuis*). The four strains of *Salmonella typhimurium* in Fig. 3 showed the same set of primary fragments at 490 and 660 bp and a weak fragment at 630 bp. These three fragments were common to each of the 10 strains of *Salmonella typhimurium* in the data base (Table 1). A band of variable intensity was observed at

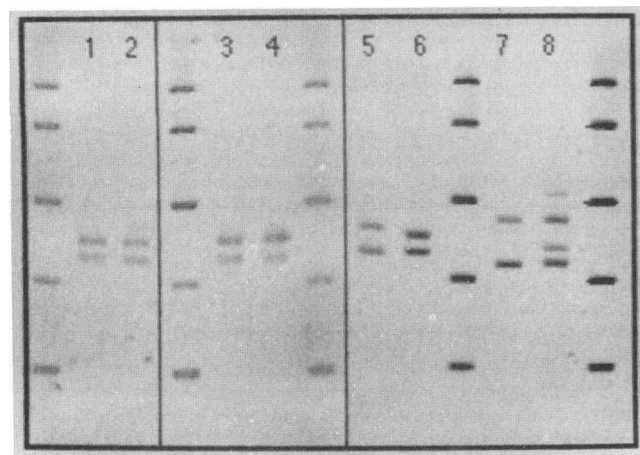


FIG. 5. Composite photograph showing PCR amplification products for the following bacterial genomic DNA samples: lane 1, *Escherichia coli* 32; lane 2, *Escherichia coli* 33; lane 3, *Escherichia coli* 46; lane 4, *Escherichia coli* 47; lane 5, *Escherichia blattae* 846; lane 6, *Escherichia fergusonii* 847; lane 7, *Escherichia hermani* 848; lane 8, *Escherichia vulneris* 850. The unnumbered lanes contained molecular weight markers having the following sizes: 228, 412, 693, 1,331, and 2,306 bp. The electrophoresis and staining conditions used are described in the legend to Fig. 2.

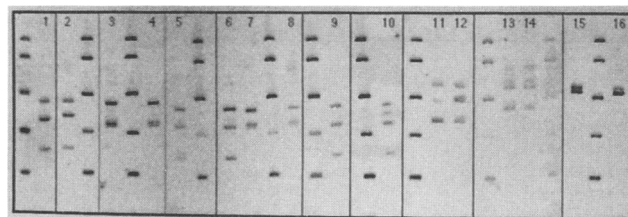


FIG. 6. Composite photograph showing PCR amplification products for the following bacterial genomic DNA samples: lane 1, *C. freundii* 355; lane 2, *C. freundii* 370; lane 3, *C. diversus* 217; lane 4, *C. diversus* 378; lane 5, *Enterobacter aerogenes* 62; lane 6, *Enterobacter aerogenes* 376; lane 7, *Enterobacter agglomerans* 901; lane 8, *Enterobacter agglomerans* 905; lane 9, *Enterobacter cloacae* 221; lane 10, *Enterobacter cloacae* 319; lane 11, *P. mirabilis* 374; lane 12, *P. mirabilis* 746; lane 13, *P. vulgaris* 930; lane 14, *P. vulgaris* 904; lane 15, *Y. enterocolitica* 750; lane 16, *Y. enterocolitica* 592. The unnumbered lanes contained molecular weight markers having the following sizes: 228, 412, 693, 1,331, and 2,306 bp. The electrophoresis and staining conditions used are described in the legend to Fig. 2.

510 bp in five strains of *Salmonella typhimurium*. Examples of *Salmonella typhimurium* producing the 510-bp band are shown in Fig. 3, lanes 3 and 4. Nine of the *Salmonella typhimurium* strains in the data base also showed a weak pair of bands at 840 and 1,150 bp. Both strains of *Salmonella enteritidis* in Fig. 3 showed common primary fragments at 480 and 665 bp. The much weaker band at 610 bp was seen in two of the four *Salmonella enteritidis* strains in the data base. Both strains of *Salmonella newport* showed common fragments at 475, 575, and 620 bp. A much weaker band could be seen 495 bp. Both strains of *Salmonella infantis* in Fig. 3 showed three common strong fragments at 490, 550, and 665 bp and two weaker bands at 510 and 620 bp. Both strains of *Salmonella typhi* showed the same set of fragments at 470 and 660 bp. The single strain of *Salmonella choleraesuis* showed two fragments at 470 and 625 bp.

Despite the similarities in the 16S-23S spacer amplification product sizes, each of the serotypes examined could be differentiated by its profile. Differentiation was made possible both by the size combinations of the two stronger fragments and by the frequent occurrence of weaker characteristic serotype fragments. Among strains of the previously recognized three species of *Salmonella*, *Salmonella choleraesuis*, *Salmonella typhi*, and *Salmonella enteritidis* (14), *Salmonella choleraesuis* was distinguished by a strong band at 625 bp, and *Salmonella typhi* was distinguished from *Salmonella enteritidis* by a 10-base difference in the shorter spacer amplification product and by a very weak 610-bp fragment seen in some strains of *Salmonella enteritidis*.

**Staphylococcus species.** Figure 4 shows the patterns of 16S-23S spacer amplification products for four species belonging to the genus *Staphylococcus*. The examples shown in Fig. 4, lanes 1 through 6, were representative of some of the different profiles generated by the amplification of this spacer region in *Staphylococcus aureus*. The diversity of these profiles indicates that for *Staphylococcus aureus* the size of this spacer region exhibits a very significant degree of intraspecies variation. The pattern in lane 2 was the most commonly observed *Staphylococcus aureus* profile, appearing in 6 of the 29 *Staphylococcus aureus* strains which were studied (Table 1). The patterns in lanes 3, 4, and 6 each appeared in three strains of *Staphylococcus aureus*. Within the *Staphylococcus aureus* data base there were a number of

TABLE 2. Summary of sizes of spacer amplification products by species

Species	Primary fragments found in most strains (bp)	Weaker secondary fragments (bp)
<i>L. monocytogenes</i>	355, 620	560, 770
<i>L. welshimeri</i>	355, 635	825
<i>L. innocua</i>	355, 655	
<i>L. ivanovii</i>	380, 605	
<i>L. murrayi</i> <sup>a</sup>	335, 570	
<i>Salmonella typhimurium</i>	490, 660	510, 630, 840, 1,150
<i>Salmonella enteritidis</i>	480, 665	610
<i>Salmonella newport</i> <sup>a</sup>	475, 575, 620	495
<i>Salmonella infantis</i>	490, 550, 665	510, 620
<i>Salmonella typhi</i> <sup>a</sup>	470, 660	
<i>Salmonella choleraesuis</i> <sup>a</sup>	470, 625	
<i>Staphylococcus warneri</i>	505	455, 525, 545
<i>Staphylococcus saprophyticus</i>	470	550, 640
<i>Staphylococcus epidermidis</i>	390	440, 510, 600
<i>Staphylococcus aureus</i>	425 (34), 520 (55), 565 (52), 620 (62), 705 (48) <sup>b</sup>	1,300–1,700
<i>Escherichia coli</i>	480, 540	590, 1,100–1,400
<i>Escherichia blattae</i> <sup>a</sup>	475, 570	
<i>Escherichia fergusonii</i> <sup>a</sup>	490, 545	
<i>Escherichia hermani</i> <sup>a</sup>	460, 605	
<i>Escherichia vulneris</i> <sup>a</sup>	460, 605, 510, 725	
<i>C. freundii</i>	320, 490 and/or 510, 620	
<i>C. diversus</i>	455, 480, 620	505
<i>Enterobacter aerogenes</i>	285, 460, 585	
<i>Enterobacter agglomerans</i> <sup>a</sup>	465, 570	445
<i>Enterobacter cloacae</i>	280 or 300, 460	510, 530, 600
<i>P. mirabilis</i>	505, 665, 850	
<i>P. vulgaris</i>	590, 840, 930, 1,200	1,070
<i>Y. enterocolitica</i> <sup>a</sup>	745, 815	

<sup>a</sup> Species for which only one or two strains were evaluated.<sup>b</sup> The numbers in parentheses are the percentages of strains in which the fragments were found.

characteristic amplification product sizes which were dispersed throughout a significant portion of the 29 strains. Some of the more frequently observed fragment sizes are as follows: 425 bp in 34% of the strains, 520 bp in 55% of the strains, 565 bp in 52% of the strains, 620 bp in 62% of the strains, and 705 bp in 48% of the strains. Approximately 40% of the strains of *Staphylococcus aureus* also showed faint bands in the range from 1,300 to 1,700 bp. Examples of such fragments can be seen in Fig. 4, lanes 1, 2, and 8. Both *Staphylococcus warneri* strains showed a primary fragment at 505 bp and three weaker fragments at 455, 525, and 545 bp. This pattern was observed for each of the five strains of *Staphylococcus warneri* in the data base. Both strains of *Staphylococcus saprophyticus* in Fig. 4 showed a primary fragment at 470 bp and two weaker fragments at 550 and 640 bp. This pattern was common to each of the eight *Staphylococcus saprophyticus* strains in the data base. Both *Staphylococcus epidermidis* strains showed a primary fragment at 390 bp and one weaker fragment at 600 bp. The 390- and 600-bp fragments were present in all five strains of *Staphylococcus epidermidis* in the data base. The two weaker fragments at 440 and 510 bp were seen in four of the *Staphylococcus epidermidis* strains.

Amplification of the 16S-23S spacer region of *Staphylococcus* species does not produce any single unifying profile feature for this genus. A corresponding level of spacer

diversity is also observed for the species *Staphylococcus aureus*. There are, however, a number of bands which are common to a significant portion of the strains of *Staphylococcus aureus*. These bands are distributed in a mixed fashion in various strains throughout the species. The presence of any combination of these bands is generally sufficient to distinguish *Staphylococcus aureus* from other species of the genus *Staphylococcus*. With the exception of *Staphylococcus aureus*, each of the species of the genus *Staphylococcus* is characterized by the production of a single strong fragment which is present at a greater level than the other amplification products. The sizes of these characteristic fragments are as follows: *Staphylococcus warneri*, 505 bp; *Staphylococcus saprophyticus*, 470 bp; and *Staphylococcus epidermidis*, 390 bp.

**Escherichia species.** Figure 5 shows the patterns of 16S-23S spacer amplification products for five species belonging to the genus *Escherichia*. The four *Escherichia coli* strains in Fig. 5 each showed the same pair of fragments at 480 and 540 bp. This pair of fragments was common to each of the 95 strains of typical *Escherichia coli* in the data base (Table 1). In addition, 12 *Escherichia coli* strains also produced a weaker fragment at 590 bp. An example of this fragment can be seen in lane 2. Approximately 10% of the *Escherichia coli* strains also showed extremely faint bands in the range from 1,110 to 1,400 bp. *Escherichia blattae* showed fragments of

475 and 570 bp. *Escherichia fergusonii* produced fragments of 490 and 545 bp. *Escherichia hermani* showed fragments at 460 and 605 bp. *Escherichia vulneris* produced fragments at 460, 605, 510, and 725 bp.

Most the *Escherichia* species could be easily distinguished by their amplification product profiles. However, the size differences between *Escherichia coli* and *Escherichia fergusonii* amplification products are sufficiently small that it is difficult to discriminate between these two species given the 2% limit in gel resolution.

**Related genera.** Figure 6 shows the patterns of 16S-23S spacer amplification products for eight additional species belonging to four genera which are related to the pathogenic species of interest. The two strains of *Citrobacter freundii* in Fig. 6 gave similar patterns with a pair of matching fragments at 320 and 620 bp. These two fragments were common to all 14 *C. freundii* strains in the data base (Table 1). A third fragment was observed at 510 and 490 bp for *C. freundii* 341 and 896, respectively. Of the 14 *C. freundii* strains, 3 strains contained the 510-bp fragment, 8 strains contained the 490-bp fragment, and 3 strains contained both fragments. The two strains of *Citrobacter diversus* in Fig. 6 showed two strong fragments at 455 and 620 bp. Each of the four *C. diversus* strains in the data base showed these fragments. Three of the four strains also showed a third fragment at 480 bp. Only *C. diversus* 227 showed a fourth additional band at 505 bp. The two strains of *Enterobacter aerogenes* in Fig. 6 showed matching patterns with common fragments at 285, 460, and 585 bp. Each of the four *Enterobacter aerogenes* strains in the data base produced these fragments. The two *Enterobacter agglomerans* strains showed identical patterns with primary fragments at 465 and 570 bp and a weaker secondary fragment at 445 bp. The two strains of *Enterobacter cloacae* in Fig. 6 showed similar patterns with three matching fragments at 300, 460, and 600 bp. *Enterobacter cloacae* 346 also produced an additional strong band at 530 bp. The two fragments at 280 to 300 and 460 bp were common to all 16 *Enterobacter cloacae* strains in the data base. An additional fragment in the range from 510 to 530 bp was observed in three strains of *Enterobacter cloacae*, nine strains showed an additional fragment at 600 bp, and four *Enterobacter cloacae* strains produced both fragments. The two strains of *Proteus mirabilis* in Fig. 6 showed identical patterns with fragments at 505, 665, and 850 bp. Each of the five *P. mirabilis* strains in the data base produced the 850- and 665-bp fragments. The 505-bp fragment was present in four strains. The two strains of *Proteus vulgaris* showed fragments at 590, 840, 930, 1,070, and 1,200 bp. The 590- and 840-bp fragments were present in each of the six *P. vulgaris* strains in the data base. The 930- and 1,200-bp fragments were found in four and five strains, respectively. The weak band at 1,070 bp was only seen for *P. vulgaris* 930 and 904. Both *Yersinia enterocolitica* strains in Fig. 6 showed a pair of fragments at 745 and 815 bp.

## DISCUSSION

PCR amplification of the 16S-23S spacer region by using the highly conserved flanking sequences G1 and L1, which are found in the adjacent 16S and 23S regions, produced amplification products for all 300 strains of bacteria in our data base. These products were generated by using a unified set of primers and PCR conditions without regard to the origin of the bacterial DNA. Since sizes and sequences of 16S-23S spacer regions have not been extensively characterized, it is difficult to predict how the sizes of amplification

products should vary across the entire range of prokaryotic organisms. For our panel of test organisms the majority of products generated by amplification from the G1 and L1 sites ranged in size from 280 to 850 bases. This size range is consistent with the limited sequencing data which are currently available for 16S-23S ribosomal spacer regions from a few species of bacteria (4, 9, 17, 23).

In some cases weak and variable secondary amplification products in the size range from 850 to 1,700 bases were also generated. These products were observed most frequently in the genera *Proteus*, *Salmonella*, and *Staphylococcus* and to a much lesser degree in the genus *Escherichia*. The yield for such fragments is significantly lower than the yield for fragments in the 280- to 850-base range. The source and nature of these larger fragments are not well understood, and it is not clear that they are necessarily double-stranded ribosomal DNA sequences. Because of the inconsistent nature of these fragments, they are not in themselves particularly useful in making species identifications. Their primary utility, when they do occur, may be in providing additional confirmation of an identification which is based on the primary amplification products.

Since the *Escherichia coli* *rrn* operons have been extensively characterized, it is possible to undertake a more detailed examination of the *Escherichia coli* spacer amplification products. The *Escherichia coli* genome is known to contain seven *rrn* loci (15, 16). In four loci the spacer region between 16S and 23S genes contains a single tRNA<sup>Glu</sup> gene. Each of the three remaining loci has two tRNA genes in this spacer region, tRNA<sup>Ile</sup><sub>1</sub> and tRNA<sup>Ala</sup><sub>1B</sub>. Previously published sequences indicate that the distance between G1 and L1 for *rrnB* and *rrnG* (4), which contain the single tRNA gene, and for *rrnD*\* and *rrnX* (28), each of which contains two tRNA genes, is approximately 530 bases. (*rrnD*\* and *rrnX* are hybrid operons whose origin and composition are described in detail in references 3 and 28.) Previously published data indicate that the distance between G1 and L1 for the *rrnE* operon (20), which contains a single tRNA<sup>Glu</sup> gene, is approximately 440 bases. Amplification with the G1 and L1 primers by using genomic DNA from each of 95 *Escherichia coli* strains produced fragments of 540 and 480 bp. The size of the 540-bp *Escherichia coli* amplification product shows a reasonable agreement with the size predicted for amplifications from the G1 and L1 locations in the *rrnB*, *rrnG*, *rrnD*\*, and *rrnX* operons. It is not clear whether the 480-bp fragment results from amplification of a spacer in the *rrnE* operon or from one of the other operons for which no sequencing data have been reported. No amplification product was observed in the 440-bp size range predicted for *rrnE* in any of the 95 strains of *Escherichia coli* studied by us.

The seven *rrn* loci of *Salmonella typhimurium* are known to have positions and orientations similar to those of the seven *Escherichia coli* operons. Like *Escherichia coli*, the *Salmonella typhimurium* genome contains four *rrn* operons with the tRNA<sup>Glu</sup> gene and three *rrn* operons with the combined tRNA<sup>Ile</sup><sub>1</sub> and tRNA<sup>Ala</sup><sub>1B</sub> genes. The only significant difference in the *rrn* loci is that the spacer tRNAs of *rrnB* and *rrnD* are switched (18). Considering the similarities in both the positions of the *rrn* loci and the tRNA compositions of the spacers regions, it is interesting to note that the product profiles obtained by amplifying the 16S-23S spacer regions of *Escherichia coli* and *Salmonella typhimurium* are relatively distinct. The 490-bp fragment of *Salmonella typhimurium* could conceivably result from the amplification of a spacer region in an *rrn* operon similar to the one that produces the 480-bp fragment in *Escherichia coli*. However, the second

primary amplification product of *Salmonella typhimurium*, the 660-bp fragment is substantially larger than the 540-bp fragment produced in the *Escherichia coli* amplifications; presumably, the spacer region is longer in the corresponding *Salmonella rrm* loci. This observation suggests that similarities in locus position and tRNA composition do not by themselves result in similar spacer amplification products.

Although the amplification reactions were run under high-stringency conditions and with a low cycle number, multiple primary amplification products were observed for most of the species in Table 1. This suggests that intragenomic spacer length polymorphism is probably characteristic of a substantial portion of prokaryotic organisms. Even though the sample set of bacteria is too small to permit broad generalizations about the intragenomic relatedness of spacer region polymorphisms, the majority of the genera studied do appear to show some characteristic size ranges for their spacer amplification products.

All of the *Listeria* species studied have two primary amplification products that are 335 to 380 and 570 to 655 bp long. This pair of amplification products differing in size by 225 to 300 bp is a common feature for all of the species of the genus *Listeria* in our data base. All representatives of the genus *Salmonella* in the data base show a pair of strong fragments in the regions from 450 to 490 and 605 to 665 bp. This pair of fragments, differing in size by 140 to 190 bp, is a feature which is unique to the genus *Salmonella* and is not observed for any other organisms in the data base. Although there are no amplification products which are common to all *Escherichia* species, this portion of the data set is weighted very heavily with *Escherichia coli* strains. A clearer understanding of the intragenomic relatedness of the spacer region polymorphisms of the genus *Escherichia* will require a broader sampling of other species in this genus.

The most significant intra- and interspecies variations in spacer amplification products are observed with the genus *Staphylococcus*. Unlike the genera *Listeria* and *Salmonella*, amplification of the 16S-23S spacer region of *Staphylococcus* species does not produce any unifying product profile traits. At the species level *Staphylococcus aureus* does not show the single profile pattern which is characteristic of most other species in the data base. However, certain characteristic fragment sizes and combinations of fragments were frequently observed for certain strains of *Staphylococcus aureus*. The high level of intraspecies variation shown by *Staphylococcus aureus* is not seen in any of the other species of the genus *Staphylococcus*. Other *Staphylococcus* species in the data base are characterized by the production of a strong representative fragment which is present at a greater level than the other amplification products. The behavior observed for the species *Staphylococcus aureus* indicates that although a number of species show relatively homogeneous groups of amplification products, it is also possible for a single species to produce a surprising variety of spacer amplification products. It appears that species definitions based on cellular metabolism and morphology can embrace a significant degree of intraspecies polymorphism in the 16S-23S ribosomal spacer region.

The samples from members of the related genera *Citrobacter*, *Enterobacter*, and *Proteus* each generated a product profile containing elements which are characteristic of its genus. Both *Citrobacter* species produce a common fragment at 620 bases, the three *Enterobacter* species studied each have two strong amplification products in the ranges from 460 to 465 and 575 to 600 bp, and both *Proteus* species produce a common band at 840 to 850 bp. In addition, the

representative species selected from the genera *Citrobacter*, *Enterobacter*, and *Proteus* also demonstrate sufficient inter-species diversity to permit the identification of the individual species. The differences in product profiles are also sufficient to differentiate the representatives of these genera from pathogenic organisms such as *Salmonella* spp., *Staphylococcus aureus* and *L. monocytogenes*.

On the basis of the results obtained with our 300-bacterial strain test group, PCR amplification of the 16S-23S spacer region shows significant promise as a tool for the identification of taxa belonging to a wider range of bacteria. The nucleotide sequences of the G1 and L1 primers are sufficiently highly conserved among these organisms to permit PCR reactions to be carried out with a single set of reaction conditions and amplification parameters without regard to species or genus. The species of all of the genera in the test group produce characteristic amplification product profiles with a level of diversity which makes possible the identification of these bacterial species. The generic character of the amplification process provides for a simple and direct genetically based diagnostic procedure for the identification of bacteria.

#### ACKNOWLEDGMENTS

We thank Charles Robertson and Romeo Hubner for their assistance in data acquisition and analysis. We also thank Eileen Cole and James Bruce for their assistance with DNA isolation.

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